Diapause in the mosquito *Culex pipiens* evokes a metabolic switch from blood feeding to sugar gluttony

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A key characteristic of overwintering dormancy (diapause) in the mosquito *Culex pipiens* is the switch in females from blood feeding to sugar gluttony. We present evidence demonstrating that genes encoding enzymes needed to digest a blood meal (*trypsin* and a *chymotrypsin-like protease*) are down-regulated in diapause-destined females, and that concurrently, a gene associated with the accumulation of lipid reserves (*fatty acid synthase*) is highly up-regulated. As the females then enter diapause, *fatty acid synthase* is only sporadically expressed, and expression of *trypsin* and *chymotrypsin-like* remains undetectable. Late in diapause (2–3 months at 18°C), the genes encoding the digestive enzymes begin to be expressed as the female prepares to take a blood meal upon the termination of diapause. Our results thus underscore a molecular switch that either capacitates the mosquito for blood feeding (nondiapause) or channels the adult mosquito exclusively toward sugar feeding and lipid sequestration (diapause).

insect diapause | overwintering | sugar feeding | digestive enzymes | fat storage

Culex pipiens (L.), a temperate-zone mosquito that vectors West Nile virus, enters an overwintering dormancy (diapause) in response to short day length and low temperatures received in the fourth larval instar and early pupal stage (1–3). Only adult females enter diapause (4, 5), and they first appear in overwintering sites such as caves, culverts, and unheated basements (6) as early as August (3, 5). One of the main features of this diapause is the arrest in development of the primary ovarian follicles (1–3). In addition, a number of behavioral changes occur, including the cessation of host-seeking behavior and the concurrent increase in feeding on carbohydrate-rich nectar, rotting fruits, and other plant products, which leads to a hypertrophy of the fat body before the onset of diapause (7–9).

Females programmed for diapause can be enticed to take a blood meal under laboratory conditions if they are placed in close proximity to a host (10–12), but most of the blood ingested by such females is ejected (12). Blood that remains in the midgut is not used to increase lipid reserves, and only a few females have been observed to use this blood to initiate vitellogenesis (12). Diapausing *C. pipiens* females are not responsive to the host-attractant lactic acid (13, 14), which suggests that the lack of blood feeding is associated with a shutdown of the host-seeking response. Thus, the arrest in ovarian development observed in diapausing *C. pipiens* is normally accompanied by a halt in blood feeding until diapause has been broken (15, 16). Like most adult diapauses (17), the diapause of *C. pipiens* appears to be the consequence of a shutdown in juvenile hormone (JH) synthesis by the corpora allata (18, 19).

The hypertrophy of the fat body and elevation of lipid reserves that are associated with diapause are linked to a boost in sugar feeding that accompanies the entry into diapause (9). The accumulation of lipid reserves occurs after adult eclosion; within a week, females programmed for diapause by short day length accumulate twice the lipid reserves of their nondiapausing counterparts, and these reserves are largely depleted during the course of the winter (8).

Although the physiological and ecological aspects of blood and sugar feeding in diapausing *C. pipiens* have been well described, the molecular events that contribute to this metabolic decision have not been explored. In this study, we used suppressive subtractive hybridization (SSH) to isolate three clones linked to this metabolic decision: *fatty acid synthase*, *trypsin*, and *chymotrypsin-like serine protease*. We then used these clones to probe the metabolic pathways associated with the mosquito's metabolic decision to enter and terminate diapause. Because both short day length and low temperature program diapause in *C. pipiens*, we also distinguished between temperature and photoperiodic effects. We concluded that the short-day programming of diapause results in the down-regulation of the genes that encode the blood-digestive enzymes and the up-regulation of a gene associated with sugar feeding and lipid sequestration.

Materials and Methods

Insect Rearing. An anautogenous colony of *C. pipiens* (L.) was established in September 2000 from larvae collected in Columbus, OH (Buckeye strain). The colony was maintained at 25°C, 75% relative humidity, with a 15 h light/9 h dark daily light/dark cycle. Eggs and first-instar larvae were kept under colony conditions until the second instar. At that time, larvae were kept in the colony rearing room (nondiapause, 25°C), moved to an environmental chamber at 18°C, 75% relative humidity, with a 15 h light/9 h dark daily light/dark cycle (nondiapause, 18°C), or placed in an environmental room under the diapause-inducing conditions of 18°C, 75% relative humidity, with a 9 h light/15 h dark daily light/dark cycle (diapause, 18°C).

Larvae were reared in dechlorinated tap water in $18 \times 28 \times 5$ cm plastic containers, fed a diet of Tetramin ground fish food (Tetra, Blacksburg, VA), and maintained at a density of ≈250 mosquitoes per pan. Adults were kept in $30.5 \times 30.5 \times 30.5$ cm screened cages and provided constant access to water and honey-soaked sponges. Honey sponges were removed from short-day cages 10–13 days after adult eclosion to mimic the absence of sugar in the natural environment during the overwintering period. None of the mosquitoes used in these experiments were offered a blood meal. To confirm diapause status, primary follicle and germarium lengths were measured, and the stage of ovarian development was determined as described in refs. 3 and 20.

SSH. Total RNA was isolated from pools of 20 females by grinding with 4.5-mm, copper-coated spherical balls in 1 ml of TRIzol

Conflict of interest statement: No conflicts declared.

Abbreviations: SSH, suppressive subtractive hybridization; DIG, digoxigenin; JH, juvenile hormone.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AY958426 (*trypsin*), AY958427 (*chymotrypsin-like serine protease*), and AY958428 (*fatty acid synthase*)].

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reagent (Invitrogen). After homogenization, samples were spun at 12,404 × *g* at 4°C for 10 min, and the supernatant was used for RNA extraction following standard protocol (21). RNA pellets were stored in absolute ethanol at −70°C and dissolved in 30 μl of ultraPURE water (GIBCO) for use in cDNA synthesis (BD SMART PCR cDNA Synthesis Kit, BD Biosciences, San Jose, CA) following standard protocol. SSH was performed by using the PCR-Select cDNA Subtraction kit (Clontech): the forward-subtracted library was constructed by using females in early diapause (7–10 days after adult eclosion, short day length, 18°C), and the reverse-subtracted library was constructed by using nondiapausing females (7–10 days after adult eclosion, long day length, 18°C). Forward and reverse libraries were cloned by using the TOPO TA Cloning Kit (Invitrogen). Transformed plasmids were inserted into competent *Escherichia coli* cells and grown overnight on Luria–Bertani (LB) plates containing X-Gal and ampicillin. For each library, >100 white colonies were isolated and grown overnight in LB–ampicillin broth at 37°C. Colonies were then purified with QIAprep Spin Miniprep (Qiagen, Valencia, CA) and sequenced by using the vector internal primer sites (T7 and M13R) at the Ohio State University Plant-Microbe Genomics Facility on an Applied Biosystems 3730 DNA Analyzer by using BigDye Terminator Cycle Sequencing chemistry (Applied Biosystems) according to manufacturer's protocol.

Northern Blot Analysis. RNA was extracted from adults and pupae according to the methods described above. Pupae were sexed as females based on their large size and prolonged development. Fifteen micrograms of denatured total RNA samples was separated by electrophoresis on a 1.4% agarose denaturing gel (0.41 M formaldehyde, 1× Mops–EDTA–sodium acetate). Visualization of ethidium bromide-stained rRNA under UV-light exposure was used to confirm equal loading. According to TURBOBLOTTER Rapid Downward Transfer Systems protocol (Schleicher & Schuell), the RNA was transferred for 1.5 h onto a 0.45-μm MagnaCharge nylon membrane (GE Osmonics, Minnetonka, MN) with downward capillary action in a 3 M NaCl/8 mM NaOH transfer buffer, followed by neutralization in a 1 M phosphate buffer solution and UV crosslinking. The membrane was then air-dried and either stored at −20°C or used immediately for hybridization.

Digoxigenin (DIG)-labeled cDNA probes were developed from the three metabolically related genes generated in our forward- and reverse-subtracted SSH libraries. PCR was performed on each clone by using the SSH nested primers (PCR-Select cDNA Subtraction Kit, Clontech) according to the following parameters: 94°C for 3 min and 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 2 min, followed by a 7-min extension at 72°C and a 4°C hold. The PCR products were run on a 1% TAE agarose gel, and the band of interest was excised from any remaining vector, extracted with Ultrafree-DA (Millipore), and reamplified by PCR. *Fatty acid synthase*, *trypsin*, and *cymotrypsin-like serine protease* cDNAs were individually labeled in an overnight DIG reaction by using 100 ng of template DNA and the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science). Probes were stored at −20°C.

Hybridization was carried out overnight and was followed by stringency washes and immunological detection by using the DIG-High Prime DNA Labeling and Detection Starter Kit II according to manufacturer's protocol. Blots were then exposed to BioMax chemiluminescence film (Kodak). Each Northern blot was replicated three or more times. To confirm equal transfer of RNA, each membrane was stripped with 0.2 M NaOH/0.1% SDS and reprobed by using DIG-labeled *28S* cDNA according to manufacturer's instructions. The *28S large ribosomal RNA* gene was obtained from our SSH library and was chosen as our control because of its high intensity and consistency of expression in all stages of mosquitoes tested.

3′ and 5′ RACE. The SMART RACE cDNA Amplification Kit (Clontech) was used to perform both 5′ and 3′ RACE. For 3′ RACE, first-strand cDNA was synthesized from 5 μg total RNA by using the manufacturer's provided adapter primer. Target cDNA was then amplified by using the universal amplification primer and a forward, gene-specific primer based on the sequence of the original clone: *fatty acid synthase* (5′-AAT TAC GCC AAA CTG C AA GG-3′), *trypsin* (5′-CAA CTT CCT CTC GTC CGG TA-3′), and *chymotrypsin-like* (5′GAT GAT CTG CCC AAG GAC TC-3′). PCR consisted of a "hot start" at 94°C for 3 min and 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min, followed by an additional 7 min at 72°C.

The 5′ RACE was carried out by using two gene-specific reverse primers according to manufacturer's protocol. Template cDNA was synthesized with *trypsin* (5′-ACG TTG GAG TAA ATT C-3′) and *chymotrypsin-like* (5′-TAT CAC AAC TCT TAA TTT C-3′) reverse primers. After purification and terminal deoxynucleotidyltransferase tailing of the cDNA, PCR was used to amplify each gene by using a second set of nested, reverse gene-specific primers and the provided abridged anchor primer. The PCR gene-specific primers were as follows: *trypsin*, 5′-GGT GAC CTC GCG ATC ATA GT-3′; *chymotrypsin-like*, 5′-TCC CAA CCT TTT CGT TGA AG-3′. Two rounds of PCR were carried out according to the parameters described above. The 3′- and 5′-RACE products were cloned and double-sequenced as described above. Although several sets of gene-specific primers were used in attempts to amplify the 5′ end of *fatty acid synthase*, a full-length sequence was not obtained.

Bioinformatics Analyses. The 5′- and 3′-RACE products were edited and assembled by using DNALIMS (dnaTools) and BIOEDIT SEQUENCE ALIGNMENT EDITOR (Isis Pharmaceuticals, Carlsbad, CA). Similar sequences were identified by performing BLASTN and BLASTX searches in GenBank (www.ncbi.nlm.nih.gov). The deduced amino acid sequences were assembled, analyzed, and aligned with similar sequences by using BLASTP, the Baylor College of Medicine Search Launcher: Sequence Utilities (http://dot.imgen.bcm.tmc.edu/seq-util/seq-util.html), and BOXSHADE SERVER 3.21 (www.ch.embnet.org/software/BOX_form.html). Percent identities were obtained by blasting two sequences with the BLASTP server by using the BLOSUM62 matrix in the National Center for Biotechnology Information's web server. The full-length nucleotide sequences for *trypsin* and *chymotrypsin-like serine protease* and the 3′ end of *fatty acid synthase* were deposited in the GenBank database and assigned accession nos. AY958426, AY958427, and AY958428, respectively.

Results

Clone Identification. Three metabolically related genes were identified by SSH at the onset of diapause (7–10 days after adult eclosion): genes encoding two blood-digestive enzymes in the class of serine proteases, *trypsin* and *chymotrypsin-like serine protease*, were among the diapause-regulated genes that were putatively down-regulated (obtained from our reverse-subtracted library), and a gene with high identity to *fatty acid synthase* was putatively diapause up-regulated (obtained from our forward-subtracted library). The cDNA matching *trypsin* is a 267-bp fragment matching with 96% identity the complete coding sequence of *C. pipiens pallens trypsin* mRNA. This portion of the clone corresponds to an 88-residue deduced amino acid sequence that matches *C. pipiens pallens* amino acids 1–76. The second clone, encoding a *chymotrypsin-like* gene, was also identified in the reverse-subtracted (down-regulated) library. The *chymotrypsin-like* clone is 294 bp long and encodes a deduced amino acid sequence of 97 residues, with 40% identity and 51% positive matches to a *serine protease* from *Anopheles gambiae*. The forward-subtracted library (up-regulated) yielded a 230-bp cDNA fragment with 85% identity to an *Armigeres subalbatus fatty acid synthase* mRNA expressed sequence tag. The deduced amino acid sequence is 76 residues long and matches

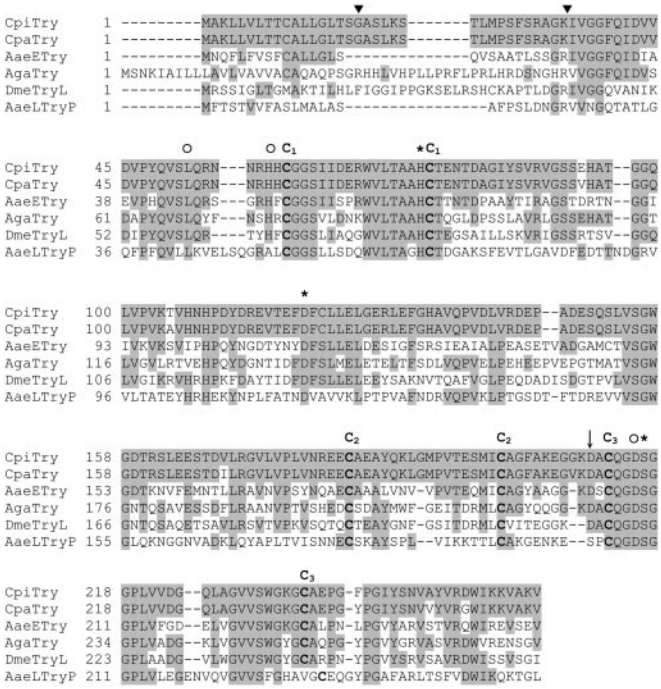

Fig. 1. Multiple sequence alignment of the deduced *C. pipiens* trypsin with other insect trypsins retrieved from GenBank. Amino acids identical to *C. pipiens* are shaded. The predicted cleavage sites of the signal peptide and the putative activation peptide are denoted with a triangle (▼). The three pairs of conserved cysteines are bolded and labeled C_1–C_3. The residues of the catalytic triad (His/Asp/Ser) are denoted by an asterisk (*), and the Asp residue characteristic of trypsin-like serine proteases is marked with an arrow (↓). Open circles (○) denote the three residues that make up the zymogen triad (Ser/His/Asp). CpiTry, *C. pipiens* trypsin, AY958426; CpaTry, *C. pipiens pallens* trypsin, AAK67462; AaeETry, *A. aegypti* early trypsin, AAM34268; AgaTry, *A. gambiae* trypsin, CAA80518; DmeTryL, *D. melanogaster* trypsin-like protease, AAC47304; AaeLTryP, *A. aegypti* late trypsin precursor, AF266757.

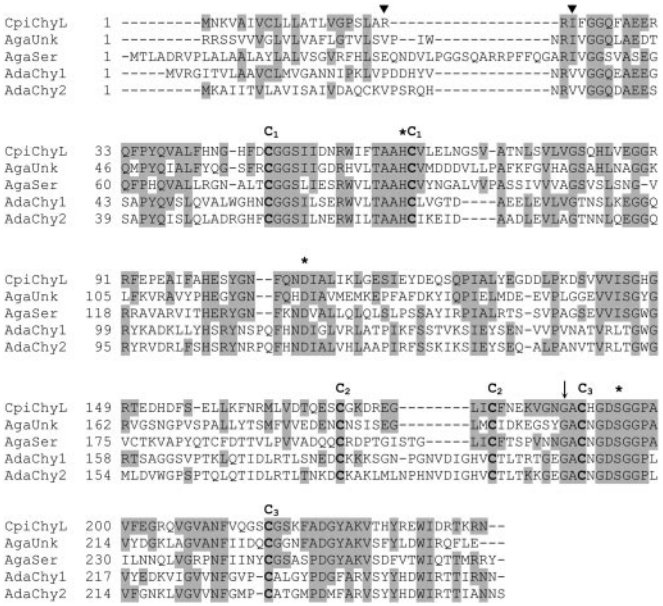

Fig. 2. Multiple sequence alignment of the deduced *C. pipiens* chymotrypsin-like serine protease with other insect trypsins retrieved from GenBank. Amino acids identical to *C. pipiens* are shaded. The predicted cleavage sites of the signal peptide and the putative activation peptide are denoted with a triangle (▼). The three pairs of conserved cysteines are bolded and labeled C_1–C_3. The residues of the catalytic triad (His/Asp/Ser) are denoted by an asterisk (*), and the Gly residue characteristic of chmotrypsin-like serine proteases is marked with an arrow (↓). CpiChyL, C. *pipiens* chymotrypsin-like serine protease, AY958427; AgaUnk, *A. gambiae* unknown protein, EAA09456; AgaSer, *A. gambiae* serine protease, AAA73920; AdaChy1, *A. darlingi* chymotrypsin 1, AAD17493; Adachy2, *A. darlingi* chymotrypsin 2, AAD17494.

amino acids 2408–2468 in fatty acid synthase from *A. gambiae*. The original clones of *trypsin*, *chymotrypsin-like*, and *fatty acid synthase* were used to generate DIG-labeled probes for Northern blot hybridization, producing bands of 0.9, 0.9, and ≈8.0 kb, respectively.

To obtain full-length cDNAs of *trypsin*, *chymotrypsin-like*, and *fatty acid synthase*, the original SSH partial cDNA sequences were used to design gene-specific primers for 5′ and 3′ RACE. The resulting *trypsin* PCR products for 5′ and 3′ RACE were 397 and 501 bp, respectively. Both sequences overlapped the initial SSH clone and yielded a total product size of 898 bp with a 783-bp ORF (see Fig. 7, which is published as supporting information on the PNAS web site). Our clone has a 46- and 27-bp 5′ and 3′ untranslated region, respectively, with a putative polyadenylation signal (AATAAA) occurring at position 839. The deduced protein has an ORF starting at nucleotide 91 and is 261 residues long. Fig. 8, which is published as supporting information on the PNAS web site, shows the full-length sequence of the *chymotrypsin-like* cDNA, along with the predicted 240-residue amino acid sequence. The 5′ RACE yielded a 609-bp product, and the 3′ RACE produced a segment 435 bp long. Together they form the complete *chymotrypsin-like* cDNA, which is 881 bp with an ORF of 720 bp. The 5′ untranslated region is 47 bp long, and the 3′ untranslated region is 94 bp with a polyadenylation site occurring at position 811.

The 3′ end of *fatty acid synthase* was obtained by RACE and resulted in a 954-bp clone that encodes 48 aa (see Fig. 9, which is published as supporting information on the PNAS web site). Our clone has a large 3′ untranslated region 810 bp long. The putative polyadenylation signal was identified at position 906. A BLASTX search revealed that this segment includes a portion of the thioesterase domain, as determined in other known protein sequences (22). Although we are confident that this clone is indeed a portion of *fatty acid synthase,* attempts at 5′ RACE were unsuccessful; thus, the full-length sequencing that will provide further confirmation of identity awaits future work.

Comparison and Analysis of the Deduced Protein Sequences. The *C. pipiens trypsin* and *chymotrypsin-like* ORFs include predicted mature active peptides in the family of serine proteases, identified by the histidine, aspartic acid, and serine residues that form the characteristic catalytic triad (23). The three cysteine bridges that form the disulfide bonds essential for holding the polypeptide chains together are also conserved (Figs. 1 and 2).

A multiple sequence alignment of the deduced trypsin amino acid sequence with the sequences of other insect trypsins is shown in Fig. 1. The *C. pipiens* trypsin ORF shares 92% identity with trypsin from a close relative, *C. pipiens pallens*. When compared with the well described trypsins from *Aedes aegypti*, our sequence aligns closest with early trypsin (51%), compared with 31% identity with late trypsin. In addition, our trypsin aligns with 53% and 51% identities to an *A. gambiae* trypsin and a *Drosophila melanogaster* trypsin-like protease, respectively. The deduced amino acid trypsin sequence has a predicted activation peptide 16 aa long, assuming a signal peptide cleavage site after Gly-18 and a putative activation peptide cleavage site after Lys-34. The putative signal peptide cleavage site was determined by using the PSORT program found at http://psort.nibb.ac.jp/form2.html (24, 25), and the activation peptide cleavage site was determined from the highly conserved IVGG sequence, common at the start of most active serine protease enzymes (26, 27). Our putative active chymotrypsin-like peptide,

Fig. 3. Multiple sequence alignment of the deduced *C. pipiens* fatty acid synthase with other insect fatty acid synthases retrieved from GenBank. Amino acids identical to *C. pipiens* are shaded. CpiFas, *C. pipiens* fatty acid synthase, AY958428; AsuFas, *A. subalbatus* fatty acid synthase, AY441061; Agaunk, *A. gambiae*, EAA15087; GgaFas, *Gallus gallus* fatty acid synthase, AAA48767; PtrPFas, *P. troglodytes* predicted fatty acid synthase, XP_511758.

however, begins with the tetrapeptide IFGG. The Asp residue characteristic of trypsin-like serine proteases was also identified in Fig. 1, along with three residues (Ser/His/Asp) referred to as the "zymogen triad," a feature that may contribute to stabilization of the inactive enzyme (28).

As indicated by a BLASTP search, our full-length clone encoding the second down-regulated digestive enzyme is most similar to serine proteases with chymotrypsin activity. The serine protease ORF aligns most closely with an undescribed protein from the *A. gambiae* genome project with which it shares a 45% identity (Fig. 2). A multiple sequence alignment highlights the identities with other insect serine proteases as follows: *A. gambiae* serine protease, 45%; *Anopheles darlingi* chymotrypsin 1, 36%; and *A. darlingi* chymotrypsin 2, 37%. By using the PSORT program, we predict the cleavage site of the signal peptide to be between Ala-20 and Arg-21. This predicted signal peptide would leave a dipeptide activation segment before the tryptic cleavage at Arg-22. In addition, the conserved Gly residue characteristic of chymotrypsin-like serine proteases (29, 30) is noted in Fig. 2.

Our predicted *C. pipiens* fatty acid synthase ORF is multialigned at the 3′ end with other fatty acid synthase sequences retrieved from GenBank (Fig. 3). The *C. pipiens* fatty acid synthase-deduced amino acid sequence is 80% identical to fatty acid synthase from *A. subalbatus* and 65% identical to an undescribed expressed sequence tag from *A. gambiae.* In addition, our alignment shows 47% identity

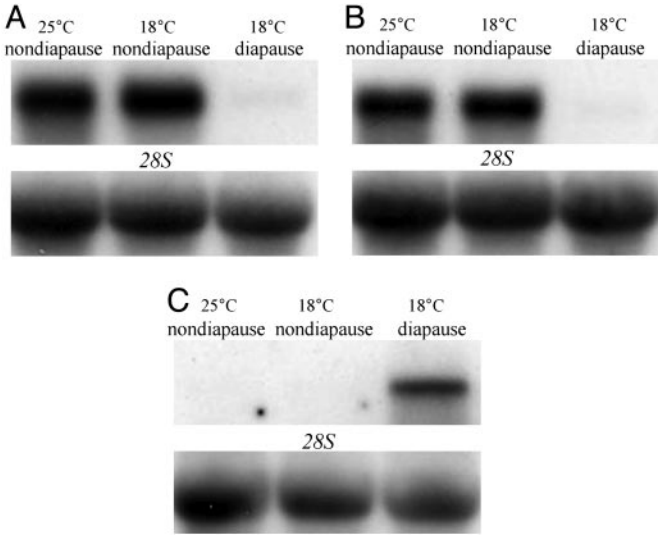

Fig. 4. Northern blot hybridization of diapause-regulated genes involved in blood meal vs. sugar meal digestion in *C. pipiens.* These Northern blot hybridizations confirm the SSH results showing early diapause down-regulation of *trypsin* (*A*) and *chymotrypsin-like serine protease* (*B*) and up-regulation of *fatty acid synthase* (*C*). The results further indicate that it is the diapause-inducing photoregime (short day length) rather than temperature that elicits the distinction. Each lane contains 15 μg of total RNA pooled from 20 females. Equal loading was confirmed by Northern blot hybridization with a *28S* cDNA probe.

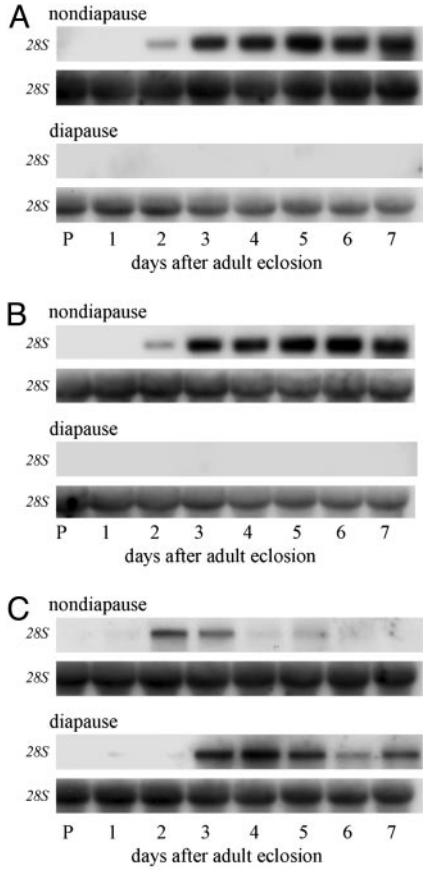

Fig. 5. Temporal pattern of expression of the genes encoding the digestive enzymes *trypsin* (*A*), *chymotrypsin-like* (*B*), and *fatty acid synthase* (*C*) in late pupae (P) and during the first 7 days after adult eclosion in nondiapausing and diapause-destined females reared at 18°C. Each lane contains 15 μg of RNA isolated from pools of 20 females. Each membrane was stripped and reprobed with DIG -labeled *28S* cDNA to confirm equal loading.

with the well described fatty acid synthase from the chicken (*Gallus gallus*) and 37% identity with the chimpanzee (*Pan troglodytes)*. Our 3′ end also overlaps a portion of the thioesterase domain as described in ref. 22.

Confirmation of Diapause Up- and Down-Regulated Genes. Northern blot hybridizations confirmed the SSH results showing the down-regulation of mRNA-encoding *trypsin* and *chymotrypsin-like* and the up-regulation of *fatty acid synthase* in early diapause (Fig. 4). Our SSH comparison used mosquitoes reared under diapause-inducing (short day length) and nondiapause-inducing (long day length) conditions at 18°C. The only environmental variable was day length, and thus we can conclude that the distinctions we observed were in direct response to photoperiod. To further evaluate the role of temperature, we also used Northern blots to compare nondiapausing mosquitoes reared at both 18°C and 25°C. The same results were observed at the two temperatures (Fig. 4); thus, the rearing temperature of the mosquitoes does not appear to be a primary environmental factor regulating the expression of *fatty acid synthase*, *trypsin*, and *chymotrypsin-like serine protease*.

Expression Patterns at the Onset of Diapause. The two blood-digestive enzymes *trypsin* and *chymotrypsin-like* have identical patterns of mRNA expression in the early days after adult eclosion (Fig. 5). In nondiapausing females, the transcripts were first detected by Northern blot hybridization 2 days after adult eclosion, and a strong signal persisted from day 3 through our final obser-

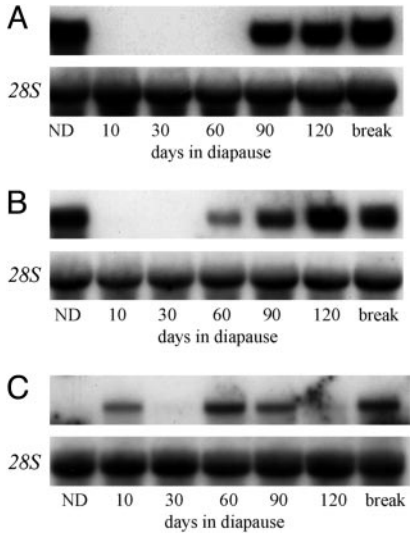

Fig. 6. Expression of *trypsin* (*A*), *chymotrypsin-like* (*B*), and *fatty acid synthase* (*C*) throughout diapause (short day length, 18°C) and when diapause is broken at 2 months. ND represents 10-day-old females reared under nondiapausing conditions (short day length, 18°C). Each lane contains 15 μg of RNA isolated from pools of 20 females. A *28S* cDNA probe was used to confirm equal loading.

vation on day 7. This pattern is consistent with the onset of host-seeking behavior in our laboratory colony; females were fully ready to take a blood meal 2–3 days after adult eclosion. By contrast, neither *trypsin* nor *chymotrypsin-like* gene expression was detectable in diapausing individuals at this early stage.

Fatty acid synthase, the gene encoding a key enzyme in the conversion of sugars to fat, was expressed at a low level in nondiapausing females, and then only on days 2 and 3 after eclosion. By contrast, this gene was highly expressed in diapausing adults beginning on day 3, and expression persisted throughout the remainder of the 7-day observation period. In all six of the independent diapause replicates, the signal was highest on day 4 and was reduced on days 5 and 6. This decrease in detectable message varied in intensity, but it was consistently observed in all replicates on day 5 or 6. Thus, in diapausing females, the onset of expression of *fatty acid synthase* occurred 1 day later, but expression was higher and persisted longer than in nondiapausing females.

Expression Patterns Throughout Diapause and at Diapause Termination. Expression of mRNAs encoding these three enzymes was also monitored throughout diapause, beginning 1 week (7–10 days) after adult eclosion and then at 30-day intervals thereafter for up to 4 months. Although *trypsin* was not expressed early in diapause (Fig. 5), a signal was evident by day 90 and persisted through day 120 (Fig. 6). A similar pattern of expression was observed for *chymotrypsin-like*, but in this case a weak signal was first noted on day 60. When diapause was broken at 2 months by transferring the females to long day length and high temperature, both genes were highly expressed within 1 week (Fig. 6).

Our Northern blots showed that the mRNA encoding *fatty acid synthase* was highly expressed in diapausing females during the first week after adult eclosion (Fig. 5), but the expression was sporadic thereafter (Fig. 6): the mRNA was consistently undetectable on day 30, strongly present on days 60 and 90, but gone again on day 120. Expression was consistently high when diapause was broken.

Discussion

SSH yielded three clones of potential interest for probing feeding responses in nondiapausing and diapausing individuals of *C. pipiens*. *Trypsin* and *chymotrypsin-like serine protease*, genes encoding two blood-digestive enzymes, are down-regulated in early diapause, and *fatty acid synthase*, a gene encoding an enzyme involved in lipid sequestration, is concurrently highly up-regulated. We have confirmed these results by Northern blot hybridization and have also demonstrated that the regulation of these genes is under photoperiodic control (short day length) and not temperature control. This is unique molecular evidence demonstrating that diapause-destined females are programmed to express a gene associated with the accumulation of lipid reserves and that these females have shut down the expression of genes associated with the digestion of a blood meal.

We obtained the 3′ end of a gene with high identity to *fatty acid synthase*, which contains a deduced amino acid sequence that overlaps a portion of the thioesterase domain of *fatty acid synthase* in *G. gallus*. As is typical of this gene, our clone also contains a long (807 bp), 3′-untranslated region with a nucleotide sequence of low homology to the other known insect *fatty acid synthase* sequences. The translated region, however, is conserved (>65%) among the three mosquito species examined. We have also identified full-length cDNA clones that encode trypsin and a chymotrypsin-like protein, two proteolytic blood-digestive enzymes in the class of serine proteases. Both deduced amino acid sequences contain the characteristic catalytic triad and the six cysteine residues typical of serine proteases (23). Our clones differ from each other, however, in the residues involved in substrate specificity. The predicted active trypsin enzyme contains a negatively charged carboxylate (Asp-210) located at the bottom of the substrate binding pocket, a typical feature of trypsin-like serine proteases, but our second clone contains a hydrophobic substrate binding pocket (Gly-189), a feature characteristic of chymotrypsin-like serine proteases. Our trypsin clone also contains the zymogen triad, three residues (Ser/His/Asp) that may help to stabilize the inactive serine protease proenzyme (28). However, our chymotrypsin-like serine protease contains only one of the three zymogen triad residues in the conserved location, a feature similar to a chymotrypsin-like protease from the human malaria vector *A. gambiae* (26). Our first serine protease clone thus appears to be a trypsin-like serine protease, and our second clone is most similar to a chymotrypsin-like serine protease.

The expression of these three genes investigated by Northern blot hybridization revealed distinct patterns of expression at the onset of diapause, during 4 months in diapause, as well as at diapause termination. During the first 7 days of adult life in diapause-destined females, *fatty acid synthase* is more highly expressed than in nondiapausing individuals, and the expression persists for a longer period. These results are consistent with the pattern of sugar feeding in *C. pipiens* (9); diapause-destined females feed on sugar more readily and for a longer period than their nondiapausing counterparts during the first 15 days of adult life. Although *fatty acid synthase* is undetectable 1 month into diapause, it is sporadically expressed thereafter until diapause has been broken. In contrast, the genes encoding the blood-digestive enzymes trypsin and chymotrypsin-like are completely "shut down" at the onset of diapause and remain down-regulated until mid to late diapause, when females are preparing for the termination of diapause.

In nondiapausing females, the up-regulation of *trypsin* and *chymotrypsin-like* 2–3 days after adult eclosion corresponds with the expression patterns of *chymotrypsin* (31) and *early trypsin* (32, 33) in nondiapausing individuals of *A. aegypti*. Because our mosquitoes were not fed blood, we would expect the *trypsin* we observed to be most similar to *early trypsin* in *A. aegypti* because *early trypsin* mRNA is abundant before blood feeding (32). The translation of *early trypsin* upon blood feeding is essential in activating the transcription of *late trypsin*, the major midgut endoprotease (33). Indeed, our *trypsin* aligns most closely with *early trypsin* in *A. aegypti* (51%), compared with 31% for *late trypsin* (31%). In addition, our *chymotrypsin-like* clone is present before blood feeding and is likely to be involved in blood digestion. Jiang *et al.* (31) characterized a female-

specific *chymotrypsin* from *A. aegypti* and demonstrated the accumulation of mRNA 24 h after adult eclosion with translation being induced after blood feeding. Unlike early trypsin, chymotrypsin remains highly active during blood meal digestion in *A. aegypti*.

Our results suggest that it is unlikely that diapause-destined *C. pipiens* females take a blood meal before entering hibernation because the molecular machinery necessary to process the blood meal is not functional in these individuals. Because the transcriptional regulation of *early trypsin* is under the control of JH in *A. aegypti* (33), the absence of *trypsin* and *chymotrypsin-like* mRNA in diapause-destined *C. pipiens* is likely a result of a lack in JH at that time. In *A. aegypti*, abdominal ligations 1 h after adult eclosion lead to a complete inhibition of *early trypsin* transcription (33). Instead of blood feeding, prehibernating females feed on nectar and other plant products early in diapause and have the ability to convert the carbohydrates into extra lipid reserves, a feature that is essential for survival throughout the long winter.

Once females enter diapause, *fatty acid synthase* continues to be expressed in mid to late diapause; thus, females are likely to be capable of processing a sugar meal throughout diapause. This continued expression of *fatty acid synthase* raises the possibility that *C. pipiens* may search for and use a readily available sugar meal during the winter. Several reports have noted that diapausing females are active even in mid-winter and often leave their hibernaculum during this time (4, 5, 34, 35). We have also observed mid-winter movement in our field sites and have observed that our laboratory-reared females will readily take a sugar meal when honey-soaked sponges are placed in their cages 2–3 months after the onset of diapause. An occasional sugar meal in mid-winter would enable females to replenish lipid reserves and may enhance survival.

As the end of diapause approaches, the accumulation of *trypsin* and *chymotrypsin-like* mRNA indicates that females are preparing for blood feeding and subsequent egg production. This pattern follows the gradual increase of JH observed in diapausing females; by the end of winter JH titers reach levels equivalent to those observed in nondiapausing females (19). It is evident from the patterns of gene expression that regulation of *fatty acid synthase*, *trypsin*, and *chymotrypsin-like* is a part of the diapause program and not a result of other factors such as temperature, host availability, or feeding activities. It has been suggested that an occasional warm spell (Indian summer) in the fall may lead *C. pipiens* to take a blood meal (10). But previous behavioral data (7, 9, 13), together with our current molecular evidence, suggest that diapause-destined females not only lack the host-seeking response but are unable to process a blood meal. Instead, such females exposed to short day length are programmed to feed more extensively on sugar and garner extra lipid reserves for winter survival. This metabolic switch is thus a component of the diapause program.

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